

Vitamin D status modulates rat colonic M₃ muscarinic receptor characteristics and coupling to guanylate cyclase

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Received 16 March 1994

Abstract

The present studies were conducted to determine whether [³H]quinuclidinyl benzilate binding in rat colonic membranes and/or carbachol-mediated stimulation of particulate guanylate cyclase were altered by changes in vitamin D status. EC₅₀ values for the stimulation of colonic guanylate cyclase by carbachol were found to be significantly greater in vitamin D-deficient rats compared to their D-sufficient counterparts. Concomitantly, the density of receptors (*B*_{max}) were significantly lower, and dissociation constants (*K*_d) were significantly higher in D-deficient colonic membranes. In vitamin D-repleted animals, moreover, all of these aforementioned alterations were at least partially corrected.

Key words: Signal transduction; Cyclic GMP; Polyphosphoinositide; Cyclic nucleotide; Rat large intestine; Muscarinic agonist

1. Introduction

Prior studies by our laboratory [1] have demonstrated that muscarinic agonists, such as bethanechol, rapidly stimulated the membrane polyphosphoinositide signal transduction pathway in isolated colonocytes prepared from vitamin D-sufficient rats. As a consequence of the activation of this pathway, moreover, bethanechol also increased intracellular calcium, induced the translocation of protein kinase C (PKC) from the cytosolic to particulate fraction and decreased intracellular pH in these cells [1]. In contrast to these findings in vitamin D-sufficient animals, bethanechol failed to elicit any of the aforementioned biochemical responses in vitamin D-deficient animals [1]. In vivo administration of 1,25-dihydroxycholecalciferol (1,25(OH)₂D₃), the major active metabolite of vitamin D₃, for 5–7 days to vitamin D-deficient animals, however, almost completely restored the ability of bethanechol to induce these rapid colonic biochemical effects [1]. While these observations indicated that alterations in the vitamin D status of rats modulated the action of muscarinic agents on the colonic polyphosphoinositide signal transduction pathway, the mechanism(s) involved in this phenomenon were unclear.

In this regard, our laboratory has more recently demonstrated that, as assessed by pharmacologic criteria, only the M₃ subtype of muscarinic acetylcholine receptor (mAChR) was present on the surface of rat colonocytes [2]. This is of interest since M₃ receptors are known to be coupled to membrane polyphosphoinositide turnover

in other cell types [3]. Moreover, we also recently demonstrated that this pharmacologic subtype of mAChRs was coupled to particulate guanylate cyclase activation in rat colonocytes [2].

Based on these observations, it was the objective of the present experiments to determine whether alterations in the vitamin D status of rats, as in the case of membrane polyphosphoinositide turnover, also influenced the ability of muscarinic agonists to activate colonic particulate guanylate cyclase. It was also of interest to determine whether changes in the binding properties of the M₃ receptors might account for the differences in the responses of these second messenger systems to muscarinic agonists under these experimental conditions. The results of these studies and a discussion of their significance serve as the basis for the present report.

2. Experimental

Male albino Sprague–Dawley rats, initially weighing 40–60 g, were used in these experiments. All diets were obtained from ICN Biochemicals (Cleveland, OH, USA) and have previously been described in detail [1,4]. Animals were placed either on a vitamin D-sufficient diet or fed a vitamin D-deficient diet and housed in a facility devoid of fluorescent light. The dietary content of calcium and phosphorus in this diet was raised to 1.8% and 0.8%, respectively, a diet previously designed to prevent the development of calcium or phosphate deficiencies, and/or secondary hyperparathyroidism, known complications of vitamin D-deprivation [1]. After 12–15 weeks, one half of the latter group of animals received 10 ng/100 g b.wt./day of 1,25(OH)₂D₃ by osmotic mini pumps implanted s.c. (2001–2002, Alzet, Palo Alto, CA, USA) for 7 days, whereas, the others received vehicle in these pumps as described [1]. Serum 25(OH)D₃ and 1,25(OH)₂D₃ levels were measured in these animals as previously described [1,4]. For the experiments described below, animals in all three groups were fasted overnight except for water ad libitum.

Rats were sacrificed and their colons removed and flushed with cold saline containing 1 mM dithiothreitol. Colonocytes were harvested

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using a technique which combined chelation of divalent cations and mild mechanical dissociation as previously described [1,4]. The isolated cells were then suspended in 50 mM Tris-HCl buffer (pH 7.5) and homogenized, and membranes were prepared as described by Mezzoff et al. [5]. Protein was determined in these samples by the method of Bradford [6]. The radiolabeled muscarinic receptor ligand, (-)-[³H]quinuclidinyl benzilate ([³H]QNB), was used to measure receptor number and affinity as described [2]. Briefly, for the saturation of [³H]QNB binding, membranes (0.1–0.2 mg protein) were incubated with the indicated concentrations of [³H]QNB (40.2 Ci/mmol, New England Nuclear, Boston, MA) at 37°C for 45 min in 1 ml of 50 mM Tris-HCl buffer (pH 7.5). At the end of these incubations, bound and free [³H]QNB were separated by rapid vacuum filtration as previously described [2]. Non-specific binding was measured in the presence of 10 μ M atropine and subtracted from total binding to obtain specific binding [2]. Receptor binding characteristics were then determined by Scatchard analysis of specifically bound [³H]QNB as previously described [2]. For the displacement of [³H]QNB binding by muscarinic receptor antagonists, membranes (0.1–0.2 mg protein) were incubated with a fixed concentration of [³H]QNB (0.3 nM) and indicated concentrations of unlabeled atropine, pirenzepine, 4-diphenylacetoxy-N-methylpiperidine iodine (4-DAMP) or methoctramine at 37°C in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) for 45 min [2]. After incubation, samples were filtered and [³H]QNB binding determined as described above.

For measurement of particulate guanylate cyclase activity, isolated colonocytes were suspended ($\sim 10^7$ cells/ml) in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 180 mg% glucose (KRBG buffer) and incubated in 2 ml aliquots at 37°C with the agents to be tested, or appropriate vehicles for the indicated times and concentrations. The reaction was terminated and membranes prepared as described previously [2]. Guanylate cyclase activity in these preparations was then assayed under conditions that were linear with respect to time and protein utilizing a cGMP RIA kit as described [2]. Enzyme activity was calculated as pmol cGMP/min/mg protein and was expressed as a percent of control (D-sufficient).

Data were analyzed by analysis of variance (ANOVA) as previously described [2]. $P < 0.05$ was considered statistically significant.

3. Results

In agreement with prior studies from our laboratory using these diets [1,4], no significant differences were noted in the body weights of the three groups of animals (data not shown). Vitamin D-deficient animals as expected, however, had very low serum 25(OH)₂D₃ levels (< 5 ng/ml) and significantly lower serum levels of 1,25(OH)₂D₃ (22 ± 4 pg/ml, $n = 14$) compared with vitamin D-sufficient (96 ± 13 pg/ml, $n = 14$, $P < 0.01$) or 1,25(OH)₂D₃-repleted rats (101 ± 17 pg/ml, $n = 14$, $P < 0.01$).

As shown in Fig. 1, membranes prepared from isolated rat colonocytes of all three dietary groups demonstrated saturability of specific [³H]QNB binding.

Moreover, as shown in the inset of this figure, Scatchard analyses of specifically-bound [³H]QNB revealed the presence of only one specific subtype of muscarinic receptors for each of these preparations over the ligand concentration tested. In agreement with our prior studies [2], the apparent equilibrium dissociation constant (K_d) and the maximum number of binding sites (B_{max}) of the membranes prepared from vitamin D-sufficient rats were 0.50 ± 0.02 nM and 426 ± 11 fmol/mg protein, respectively (Table 1). As can be seen in this table, the K_d value was significantly higher, whereas the B_{max} value was significantly lower in membranes prepared from vitamin D-deficient rats, compared to their vitamin D-sufficient counterparts. Moreover, vitamin D-repletion was found to totally restore the decreased B_{max} values seen in D-deficient membranes to control (D-sufficient) values (Table 1). As shown in this table, however, D-repletion significantly ($P < 0.05$), but only partially, reduced the elevated K_d values seen in vitamin D-deficient colonic membranes toward control levels.

To determine whether differences in vitamin D status might lead to alterations in the pharmacological profile of mAChRs in the rat colon, we examined the ability of various selective muscarinic receptor antagonists to compete with [³H]QNB binding to rat colonic membranes prepared from animals of all three dietary groups. The antagonists used included pirenzepine (M₁-selective antagonist) [7], methoctramine (M₂-selective antagonist) [7], 4-DAMP (M₁ and M₃ selective antagonist) [8,9] as well as atropine, a non-selective muscarinic antagonist. In agreement with our prior studies [2], the binding of [³H]QNB to colonic membranes prepared from vitamin D-sufficient rats was inhibited by these antagonists with the following rank order of potencies (inhibitory constants (K_i), nM): atropine (3.0 ± 0.5) = 4-DAMP (5.1 ± 0.7) \gg pirenzepine (155 ± 48) > methoctramine (466 ± 72) (Table 1). This data is consistent with the existence of the M₃ pharmacologic subtype of mAChRs on rat colonocytes [2]. Furthermore, the K_i values obtained, and therefore the rank order of inhibitor potencies, was found to be similar in each of the three experimental dietary groups (Table 1), indicating that the vitamin D status of the animals did not alter the pharmacological profile of mAChRs in these cells.

In preliminary studies, and in agreement with our

Table 1
[³H]QNB binding properties in rat colonic membranes^a

Experimental groups	B_{max} (fmol/mg protein)	K_d (nM)	K_i (nM)			
			Atropine	Pirenzepine	4-DAMP	Methoctramine
Vitamin D-sufficient	462 ± 11	0.50 ± 0.02	3.0 ± 0.5	155 ± 48	5.1 ± 0.7	466 ± 72
Vitamin D-deficient	$201 \pm 10^*$	$0.88 \pm 0.02^*$	2.6 ± 0.7	183 ± 36	6.7 ± 1.1	526 ± 98
Vitamin D-repleted	431 ± 34	$0.70 \pm 0.02^*$	2.8 ± 0.8	171 ± 42	6.3 ± 0.8	539 ± 84

^a Values represent mean \pm S.E.M. of four separate experiments for each experimental group.

* $P < 0.05$ compared to vitamin D-sufficient values.

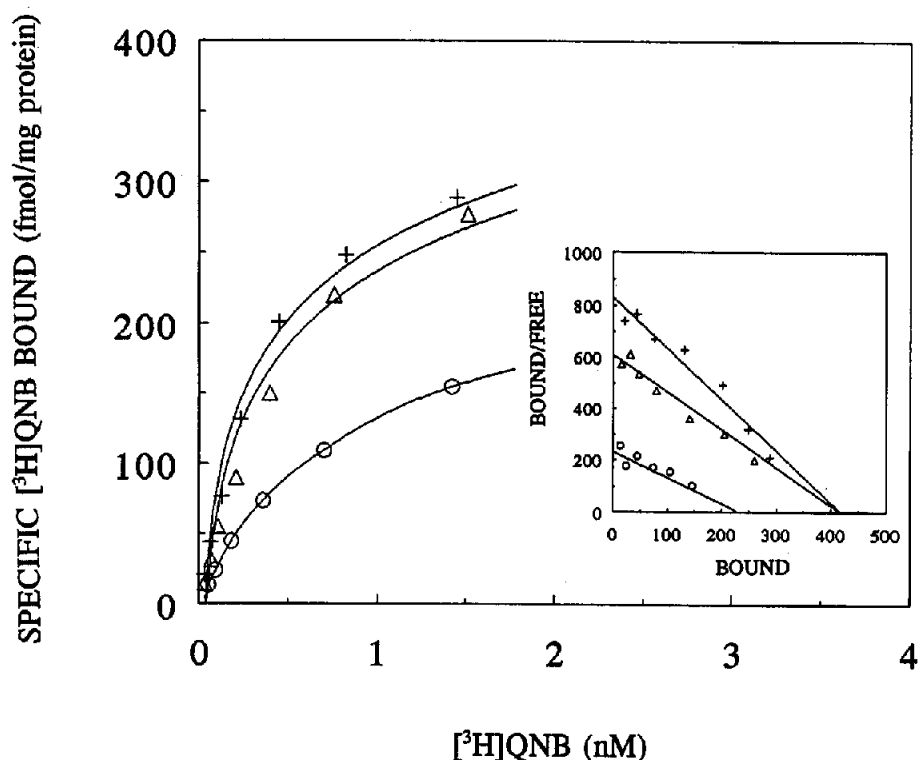


Fig. 1. Saturation of specific [^3H]QNB binding to rat colonic membranes. Aliquots of colonic membranes prepared from vitamin D-sufficient (+), D-deficient (o) and $1,25(\text{OH})_2\text{D}_3$ -repleted (Δ) animals were incubated with the indicated concentrations of [^3H]QNB for 45 min at 37°C . Non-specific binding was measured in the presence of $10\text{ }\mu\text{M}$ atropine and subtracted from total binding to obtain specific binding. Inset: Scatchard analysis of the data with the units of bound/free being fmol/mg protein/nM, and the units of bound being fmol/mg protein. Each point represents the mean of four separate experiments performed in triplicate for each experimental group.

prior findings in vitamin D-sufficient rats [2], the addition of carbachol (10^{-4} M , final concentration) to isolated colonocytes prepared from all three dietary groups of animals increased particulate guanylate cyclase activity maximally after 45 s (data not shown). Based on these findings, concentration-dependent studies were performed, using doses of carbachol ranging from 0.01 to $1,000\text{ }\mu\text{M}$, in order to obtain EC_{50} values (the concentration of agonist which increases colonic particulate guanylate cyclase activity by 50%) in each of the three dietary groups. The EC_{50} values for the stimulation of guanylate cyclase by carbachol were significantly higher ($P < 0.01$) in colonic membranes prepared from D-deficient animals ($7.4 \pm 1.1\text{ }\mu\text{M}$, $n = 6$) compared to their D-sufficient ($0.4 \pm 0.1\text{ }\mu\text{M}$, $n = 6$) and D-repleted ($1.6 \pm 0.2\text{ }\mu\text{M}$, $n = 6$) counterparts. Moreover, the maximal activation of particulate guanylate cyclase by carbachol (1 mM , final concentration) in vitamin D-deficient and vitamin D-repleted preparations was $40 \pm 3\%$ and $74 \pm 6\%$, respectively, of control (D-sufficient) values.

4. Discussion

The present studies demonstrate for the first time that

vitamin D-deficiency leads to an approximately 50% reduction in M_3 receptor density as well as a statistically significant decrease in the affinity of these receptors for muscarinic agonists in rat colonic membranes. Moreover, in the vitamin D-deficient preparations, the efficacy of muscarinic agents to activate particulate guanylate cyclase is reduced. In addition, we noted a decreased potency of carbachol, as measured by the increase in the EC_{50} values, to activate particulate guanylate cyclase in colonocytes prepared from vitamin D-deficient rats. While these phenomena may, at least in part, be related to the aforementioned alterations in the binding properties of the M_3 receptors, these alterations in binding would not fully explain the total inability of carbachol to stimulate polyphosphoinositide turnover previously noted in these cells [1]. These findings, rather would indicate additional effects of vitamin D deficiency at the post-receptor level.

In this regard, M_3 mAChRs, are known to interact with heterotrimeric guanine nucleotide-binding (G) proteins to stimulate phosphoinositide phospholipase C and, thereby, increase the turnover of membrane polyphosphoinositides [10]. Moreover, since the M_3 subtype, like other subtypes of mAChRs can couple to multiple G proteins to induce different transduction events [11], it would not appear unreasonable to suggest that the

activation of particulate guanylate cyclase by carbachol might also involve a G protein.

While the mechanism(s) involved in the effects of vitamin D-deficiency on colonic receptor- and post-receptor-mediated signal transduction events are unclear, they may involve activation of PKC. In prior studies, our laboratory has demonstrated that PKC appears to be constitutively activated in colonocytes prepared from vitamin D-deficient rats, as assessed by changes in the subcellular distribution of this serine/threonine kinase [1] and its ability to phosphorylate membrane proteins [12]. Moreover, this colonic activation of PKC in D-deficient animals was corrected by vitamin D repletion [1,12]. These observations are of particular interest in view of prior studies in neuroblastoma cells which have demonstrated that phorbol esters, which activate PKC, reduced the number of mAChRs [13–15], decreased the affinity of mAChRs for agonists [16], as well as attenuated receptor-mediated stimulation of polyphosphoinositide hydrolysis [17,18] and cGMP production [19]. While it has been proposed [20] that these actions of PKC in neuroblastoma cells involve phosphorylation of mAChRs, this has not been directly demonstrated. More recently, however, Richardson et al. [21], using mAChRs purified from chicken heart, demonstrated that PKC could phosphorylate mAChRs which, in turn, modulated the receptor/G-protein interaction in an *in vitro* system. Based on these observations, further studies to determine whether PKC is involved in the alterations in muscarinic receptor-mediated events noted in colonocytes of vitamin D-deficient rats will be of interest.

Finally, the studies of Latifpour et al. [22] deserve comment. These investigators, using the streptozotocin (STZ) model of experimental diabetes, reported that the B_{\max} of M_3 mAChRs in prostate plasma membranes prepared from diabetic rats was significantly decreased, but that insulin could prevent this receptor's down-regulation [22]. This is of considerable interest in view of earlier studies by our laboratory [23] and others [24], which documented a decrease in the serum levels of $1,25(\text{OH})_2\text{D}_3$ in animals treated with STZ, secondary to decreased renal 1α -hydroxylation. Insulin therapy, moreover, has been shown to prevent or correct these alterations in $1,25(\text{OH})_2\text{D}_3$ levels in this model [23,24]. It is, therefore, theoretically possible that alterations in the density of M_3 receptors in the prostatic membranes of these animals was due, at least in part, to differences in their vitamin D status. Based on these findings and the present observations, it will, therefore, be of interest to determine in future studies whether the vitamin D status of rats can influence mAChRs-mediated signal transduction events in other cell types besides colonocytes.

Acknowledgements: We wish to thank Lynn Nelson for her excellent secretarial assistance. This work was supported by USPHS Grant DK39573, USPHS Grant CA36745, USPHS Grant P-30-DK42086 (Digestive Diseases Research Center) and USPHS Grant P30-DK26687 (Clinical Nutrition Research Unit).

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